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LONG TERM CRYOPRESERVATION OF DOG GRANULOCYTES.(U)
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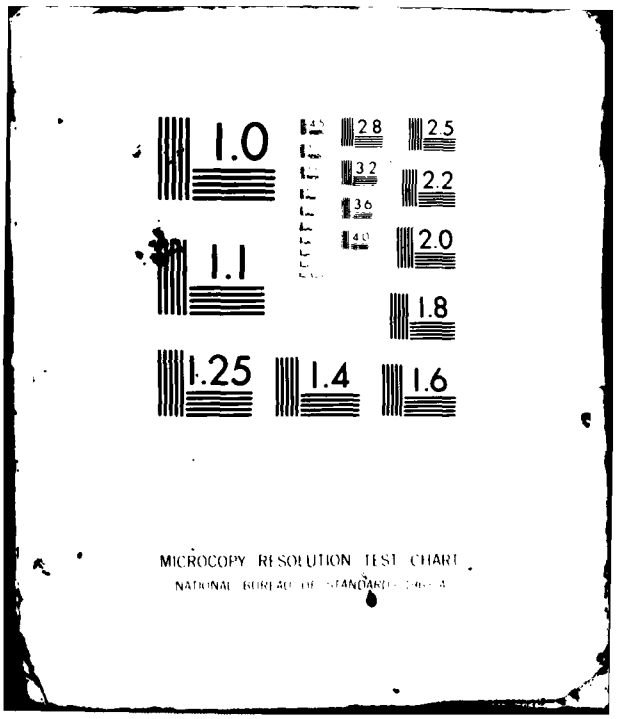
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LONG TERM CRYOPRESERVATION OF DOG GRANULOCYTES.

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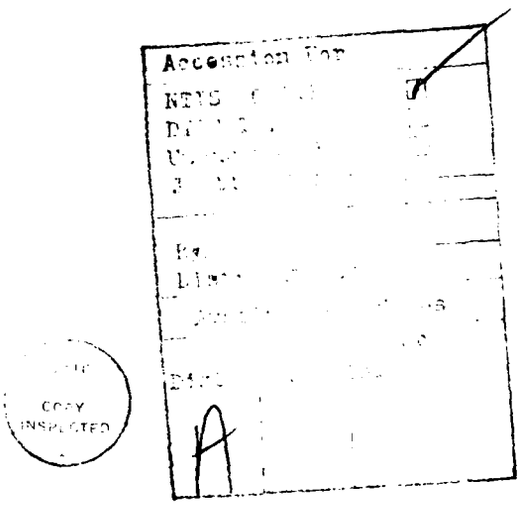
LONG TERM CRYOPRESERVATION OF DOG GRANULOCYTES

ABSTRACT

Granulocytes isolated by counterflow centrifugation eluriation (CCE) from leukapheresed dog blood, frozen in liquid nitrogen at -196°C, were studied. The effects of long term cryopreservation on cell recovery and in vitro function were determined. In eight separate experiments, an average of 1.7×10^9 granulocytes were obtained. The white cell differental count was 91% granulocytes and 9% mononuclear cells. There was less than 5% red cells present and no platelets. The granulocytes were placed in Hemoflex bags and mixed slowly with equal volumes of sterile ice-cold hyperosmolar cryoprotectant buffer to make a final composition of 5% dimethysulfoxide (DMSO), 6% hydroxyethylstarch (HES) and 4% bovine serum albumin (BSA), pH 7.1. A total volume of forty ml was frozen at a cooling rate of 4°C per minute by storage in a mechanical freezer at -80°C. After storage periods of 1, 34, 60, 90 and 132 weeks in liquid nitrogen at -196°C, a bag was thawed at a rate of 190 degrees per minute to 10°C. The recovery of cells at these periods was 95%, 105% 100%, 100% and 88% respectively and the ethidium bromide exclusion, indicative of viable nuclei, was 91%, 81%, 94%, 89% respectively. Ingestion of opsonized Fluolite particles was measured and virtually all cells ingested particles but the number ingested was approximately one half that of prefreeze values. Thawed cells also demonstrated superoxide anion synthesis at rates approximating those in unfrozen granulocytes. These results indicate that dog granulocytes obtained by leukapheresis may be preserved in liquid nitrogen at

196°C with high cellular recovery and at least 50% phagocytic function.

Key Words: Granulocytes, Cryopreservation, Elutriation



INTRODUCTION

Cryopreservation of human granulocytes has been studied by many investigators but successful cryopreservation of cell numbers sufficient for a therapeutically effective transfusion has yet to be achieved. Investigation with granulocytes of animals have been the most successful (1). French and associates (2) reported high cellular recoveries and high function of dog granulocyte concentrations prepared by counterflow centrifugation elutriation (CCE) and leukapheresis, using DMSO, HES and autologous plasma as the cryoprotectant. Our laboratory, employing CCE of whole blood and buffy coats, has also reported cryopreservation of dog (1), baboon (3) and guinea pig granulocytes (4) using 5% DMSO, 6% HES, and 4% bovine serum albumin (BSA) formulated in Normosol-R pH 7.4 adjusted to pH 7.1.

Leukapheresis by means of a Haemonetics Model 30 blood cell processor followed by CCE enabled us to collect granulocyte suspensions of high purity ($91 \pm 1.4\%$) and numbers (1.7×10^9). Six bags obtained from one dog over a period of one month were studied for the effects of long term (2 1/2 years) storage in liquid nitrogen on the recovery and function of dog granulocytes. Due to the small number of bags (n=6) available, we arbitrarily chose to thaw one unit at a time at 30 week intervals as long as the cells were stable. Therefore, granulocyte concentrates were thawed at time intervals of 1, 34, 60, 90, and 132 weeks before significant losses occurred. They were assessed for viability microfluorimetrically with vital stains fluorescein diacetate

and ethidium bromide, by ingestion of opsonized Fluolite particles, and by volume distributions. In later samples, the production of O_2^- anion radical was measured. In this paper we report the results of long term cryopreservation on recovery and in vitro function of dog granulocytes.

MATERIALS AND METHODS

Blood collection, isolation and cryopreservation.

Leukapheresed dog blood was obtained and processed as described earlier by Lionetti et al. (1). Briefly, 100 ml of leukapheresed dog blood was subjected to counterflow centrifugation as described (5), pooled and diluted to 20 mls with elutriation buffer (1.5% BSA, 28 mM glucose, PBS 0.01M phosphate) and aseptically placed in a Hemoflex bag (Union Carbide, Chicago, Ill., 150 ml capacity). An equal volume (20 ml) of sterile ice-cold cryoprotectant was added in 5 increments of 4 ml each. The final composition was 5% DMSO, 6% HES, 4% BSA and 56 mM glucose in Normosol-R pH 7.4 adjusted to pH 7.1. The bag was placed between stainless steel perforated plates to form a thin section and allowed to freeze at 4°C per minute cooling rate in the bottom of a -80°C Harris Mechanical Freezer. Once frozen, the bags were transferred and stored at -196°C in liquid nitrogen.

Granulocyte thawing and washing. Thawing was accomplished within 50 seconds of initiation by rapid back and forth agitation in a 37°C water bath. The bag was removed as the final ice pellets dissolved and placed in a shaking water bath at 2°C. The cells were diluted dropwise (4 ml per min.) with 3 volumes of sterile (0.22 μm Falcon filter) buffer composed of 6% HES, 4% BSA, 56 mM glucose in Normosol-R pH 7.1 at room temperature, transferred to a TA3 150 ml

Transferpack, (Fenwal Laboratories, Morton Grove, Ill.) and then centrifuged at 350 x g for 10 min. The supernatant was removed and the cells resuspended to 40 ml with the 6% HES buffer.

Determination of cell counts and size distribution. A Coulter Counter Model ZH with a C-1000 Channelyzer system was used to measure RBC and WBC counts as published by Contreras et al. (6). Cell volume was determined by calibrating the instrument with polystyrene particles of 5.13 μm and 10.14 μm diameters and 70.5 μm^3 and 544 μm^3 volumes respectively. The median channel number was determined by machine integration as the midpoint of the distribution.

Membrane integrity by microfluorescence. The integrity of granulocyte membranes was studied with the fluorescein diacetate (FDA) and ethidium bromide (EB) method published by Dankberg and Persidsky (7). Briefly, cells with intact cytoplasmic membranes hydrolyze fluorescein diacetate into free fluorescein which accumulates and fluoresces green. Intact nuclear membranes exclude ethidium bromide. Cells with damaged membranes leak fluorescein and are permeable to ethidium bromide which interacts with nuclear DNA producing intense orange-red fluorescence. Cells were viewed under an Olympus Vanox transmission fluorescence microscope equipped with a U.V. exciter filter (Schott BG-12) and a blue barrier filter (Schott OG-530).

Determination of O_2^-. Superoxide anion production was measured in a Cary 118 dual beam spectrophotometer at 550 nM set to 37°C, as the change in optical density of cytochrome-C by granulocytes stimulated with phorbol myristate acetate (PMA) as published by Cohen et al. (8).

Particle ingestion assay. Ingestion of Fluolite (a polycyclic hydrocarbon) was studied following the method described by Aranout et al. (9). Briefly, 0.5 ml of serum opsonized Fluolite particles were incubated at 37°C with 5 x 10^6 granulocyates for 30 minutes at a particle to cell ration of 75:1. Fresh cells were washed twice with ice cold 1% BSA (w/v) in PBS and frozen cells once with the 6% HES wash buffer at 350 x g for 10 min. The pellet was resuspended in 0.4 ml HBSS with Ca++ and Mg++ containing FDA and EB. Green fluorescing cells containing particles were graded microscopically at 400 x by the following criteria: 1) zero (Z) 0-5 particles, 2) low (L) 5-10 particles, 3) medium (M) 10-25 particles, 4) high (H) more than 25 particles per cell. An avidity index was arbitrarily defined as the sum of H and M.

Hydroxyethylstarch (Cryo HES, M.W. 150,000, 40% w/v in distilled water, McGraw Laboratories, Irvine California, Lot # P02303C) was prepared and used in the freezing and wash buffer as previously described by Lionetti et al. (3). Normosol-R pH 7.4 was obtained from Abbott Laboratories, North Chicago, Ill. Bovine serum albumin, fluorescein diacetate, ethidium bromide, superoxide dismutase type III, cytochrome-C type VI and phorbol myristate acetate were obtained from Sigma Chemical Co., St. Louis, Missouri. Fluolite DS-5005 was obtained from ICI, Finland. Dimethysulfoxide (DMSO) was reagent grade and obtained from Fisher Scientific Co., Medford, Mass. Hank's Balanced Salt Solution (HBSS with Ca++ and Mg++) was obtained from M.S. Bioproducts, Walkerville, Maryland. Phosphate buffered saline was made by diluting 0.11 phosphate buffer, pH 7.1, 10 times with 0.15 M NaCl.

RESULTS

Granulocyte isolation. The data in Table I represent eight experiments of granulocytes from leukapheresed dog blood. After leukapheresis, 77% ± 7% of granulocytes in the collection bags were recovered by counterflow centrifugation. Wright's stained smears indicated 91% ± 1.4% were granulocytes and 9% ± 1.4% were mononuclear cells. There was a RBC contamination of 5% ± 1% and no platelets. Ninety-nine percent of the isolated cells produced free fluorescein from fluorescein diacetate and one percent reacted with ethidium.

Granulocyte preservation. Granulocyte recoveries after thawing, diluting and incubation at 37°C were calculated by integration of the areas under volume distribution plots, and the cells present at each step expressed as a percent of the original prefreeze value, are shown in Table 2. The cells frozen as described yielded good to excellent recoveries when thawed after storage for 90 weeks (100%) and 132 weeks (88%). Losses of cells occurred at the dilution and incubation steps in all experiments, but recoveries were still greater than 63% for all samples. Five of the 6 storage studies maintained recoveries greater than 74% at the incubation step.

In the experiments with dog granulocytes (Table 2) the volumes during the manipulations of preservation are shown. It is evident from the data of Table 2 that dog granulocytes frozen one week were well preserved. In two separate bags, there was high recovery of thawed cells. These had small percentages of ethidium reactive nuclei and the volume were well maintained at the prefreeze (PF), post-thawed (PT), diluted (PTD), and incubated (PTDI) stages

Granulocytes were lost after thawing, during washing and after 37°C incubation. These losses did not become appreciable until 60 weeks of storage and thereafter. In the granulocytes stored 60, 90, and 132 weeks, progressive increases in ethidium uptake were observed indicative of unstable nuclei.

Ingestion of opsonized Fluolite particles and superoxide production (O_2^-) were both measured to determine functional properties of the cryopreserved granulocytes (Table 3). The number of granulocytes which ingested particles remained high throughout the study as approximately 80% of frozen granulocytes ingested 5 or more particles. Significantly, a change in avidity for particles was evident as the number with many particles ingested per cell (H + M) decreased approximately 50%. This appeared to be a consequence of freezing injury as the decrease in avidity (Table 3) was approximately 50% throughout the storage period. One high value (177% increase) we attribute to an experimental flaw in the high (H) index at 60 weeks.

Superoxide anion synthesis by dog granulocytes was measured in two bags, stored 90 and 132 weeks, and compared with unfrozen control granulocytes from another dog (Table 3, bottom). Granulocytes from the control dog were frozen and stored for 3 weeks. The magnitude of superoxide anion synthesis was the same (ca 3.3 nMoles/min/10^6 cells) for all samples. Niether freezing, nor storage, affected the rate.

DISCUSSION

In these studies we have demonstrated that dog granulocytes isolated, frozen and stored in liquid nitrogen at -196°C for up to 132 weeks were recovered in large quantities and had well preserved postthawed in-vitro function.

The methods of isolation, similar to those of French and associates (2), demonstrate high cell recovery with minimal damage to cell cytoplasmic and nuclear membrane as assayed microfluorimetrically with fluorescein diacetate (99% positive) and ethidium bromide (1% positive).

This study and previous studies of granulocytes (1,2,3,4) and monocytes (10) frozen with intracellular hyperosmolar cryoprotectants containing 5% DMSO and 6% HES have demonstrated the importance of controlling cell volume throughout prefreeze and postthaw steps. The use of volume measurements and vital stains on prefrozen and thawed samples enables cell recovery, volume and viability to be monitored throughout to help evaluate pre-freeze and post-thaw cell stability. Results by electronmicroscopy of frozen dog granulocytes by French and associates showed overall retention of cellular ultrastructure and phagocytic ability but also noticeable alteration in granule structure (11).

Particle ingestion and superoxide production in our studies revealed the cells to be phagocytic and biochemically intact upon thawing. The phagocytic indices, especially the capacity for ingestion, was diminished after freezing and as storage time at -196^{o}C increased. The phagocytic indices and cell recoveries decreased while the ethidium permeability increased. Comparison of superoxide anion synthesis in fresh unfrozen and postthawed washed control cells showed comparable rates of enzyme activity after storage in liquid nitrogen for 3 weeks. Similar rates were observed in cells stored at -196^{o}C for 132 weeks of the experimental dog showing great stability of the membrane enzyme(s) involved. These data show that superoxide anion synthesis is a membrane phenomenon (12) distinct from particle ingestion which is more vulnerable to preservation injury.

Granulocytes lost approximately half their capacity for particle ingestion (Table 3) after freezing and storage at -196°C for one week. Thereafter the phagocytic avidity remained about 50% of prefrozen values for 132 weeks. This suggests the loss of activity was caused by freezing and thawing and was much less affected by storage at liquid nitrogen temperatures.

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TABLE 1. RECOVERY OF GRANULOCYTES AFTER LEUKAPHERESIS AND COUNTERFLOW CENTRIFUGATION ELUTRIATION

Recovery and Cell Counts	Mean + S.E.	Range
Total leukocytes		
Initial	$2.2 \times 10^9 \pm 0.2$	$1.6\text{-}3.1 \times 10^9$
Final	$1.7 \times 10^9 \pm 0.1$	$1.3\text{-}2.2 \times 10^9$
Recovery of initial (%)	77 ——	57-110
Microfluoresence Counts (%)		
FDA	99 ± 0.1	98-100
EB	1.0 ± 0.1	0-2
WBC Differential Count (%)		
Granulocytes	91 ± 1.4	85-96
Mononuclear cells	9 ± 1.4	6-15
RBC (% of total cells)	5.3 ± 0.5	2-9

TABLE 2. RECOVERY, MEMBRANE STABILITY, AND VOLUMES OF CRYOPRESERVED DOG GRANULOCYTES

Cell Values	Number of weeks frozen					
	1	1	34	60	90	132
Recovery						
PF	—	—	—	—	—	—
PT	100	89	105	100	100	88
PTW	100	87	101	100	100	88
PTWI	100	77	87	63	74	77
Ethidium Reactive Cells						
PF	0	0	2	2	4	0
PT	6	11	19	6	11	20
PTD	8	10	21	3	9	37
PTWI	8	14	17	16	—	17
Median Channel #						
PF	43	41	43	49	47	57
PT	44	40	41	42	37	44
PTD	43	43	36	45	41	44
PTWI	42	40	39	44	45	42
Volume (μm^3)						
PT	253	241	253	288	276	335
PF	259	235	241	247	218	259
PTD	253	253	212	265	241	259
PTWI	247	238	230	259	265	247

PF is prefrozen, PT is postthawed, PTD is diluted, and PTWI is washed and incubated.
Values are averages of duplicate determinations.

TABLE 3. INGESTION OF FLUOLITE PARTICLES AND SUPEROXIDE ANION PRODUCTION BY CRYOPRESERVED GRANULOCYTES

Time Frozen (wks) at -196°C	No. of Bags	Sample	Phagocytosis: Ingestion Index (%) H	M	L	Zero	Avidity Index (%) H + M	Change in Avidity[a] (%)	Superoxide O_2^- (nm/min/10^6 cells)
1	2	PF	69	25	6	0	94	-54	—
		PT	10	39	56	0	49		—
34	1	PF	—	—	—	—	—	—	—
		PT	10	50	20	20	60		—
60	1	PF	12	39	42	7	51	+177	—
		PT	32	58	8	2	90		—
90	1	PF	32	50	16	2	82	-48	—
		PT	8	31	43	18	39		3.5 ± 0.2
132	1	PF	45	38	15	2	83	-46	—
		PT	10	28	44	18	38		4.7 ± 0.8
No. of Tubes									
Control	1	PF	81	9	8	2	90	—	[b] 3.3 ± 0.2 (3.8-2.8)
3	3	PT	18	34	47	1	52	-58	3.3 ± 0.1 (3.8-2.3)

PF is prefrozen and PT is postthawed.

a. Percent change in avidity index was calculated by dividing the postthawed by the prefrozen percentages.

b. The mean ± standard error and the range (parenthesis) is given.

Control consisted of one tube from a bag of leukapheresed blood divided into four aliquots (2.0 ml) after counterflow separation and admixture with cryoprotectant. O_2^- was measured four times.

Three tubes were frozen, stored 3 weeks, thawed, diluted and measured in duplicate.

H is high, M is medium, L is low as defined in Methods.

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Abstract continued.

slowly with equal volumes of sterile ice-cold hyperosmolar cryoprotectant buffer to make a final composition of 5% dimethysulfoxide (DMSO), 6% hydroxethylstarch (HES) and 4% bovine serum albumin (BSA), pH 7.1. A total volume of forty ml was frozen at a cooling rate of 4°C per minute by storage in a mechanical freezer at -80°C. After storage periods of 1, 34, 60, 90 and 132 weeks in liquid nitrogen at -196°C, a bag was thawed at a rate of 190 degrees per minute to 10°C. The recovery of cells at these periods was 95%, 105% 100%, 100% and 88% respectively and the ethidium bromide exclusion, indicative of viable nuclei, was 91%, 81%, 94%, 89% respectively. Ingestion of opsonized Fluolite particles was measured and virtually all cells ingested particles but the number ingested was approximately one half that of pre-freeze values. Thawed cells also demonstrated superoxide anion synthesis at rates approximating those in unfrozen granulocytes. These results indicate that dog granulocytes obtained by leukapheresis may be preserved in liquid nitrogen at 196°C with high cellular recovery and at least 50% phagocytic function.


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